

WHAT IS CLAIMED IS:

Method for simultaneous isolation of biologically active transcription factors and DNA comprising the steps of:

- 1 Collecting and maintaining cells at a concentration in the range of $1-2 \times 10^5/\text{ml}$ in culture medium,
- 2 Treating cells with at least one test compound or obtaining the cells from the treated patients,
- 3 Collecting the cells by scrapping and by centrifuging at 1,000 rpm for 5 min in a table top centrifuge,
- 4 Washing the cell pellets twice with 5 ml ice cold PBS, (phosphate buffered saline, pH 7.4) by centrifuging the cells again at 1,000 rpm,
- 5 Resuspending the cell pellets, transferring to a first Eppendorf tube in 1 ml of ice cold PBS,
- 6 Centrifuging at 2,000 rpm for 5 min at 4 °C removing the PBS processing the cell pellets following according to the protein and DNA isolation steps comprising,
- 7 Preparing Buffer A: (cell lysis buffer), [20 mM Hepes, pH 7.9, 10 mM NaCl, 3 mM MgCl_2 , 0.1% NP-40, 10% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride (PMSF), antipain (1 $\mu\text{g}/\text{ml}$), leupeptin (1 $\mu\text{g}/\text{ml}$)],
- 8 Preparing Buffer B: (extraction buffer without salt), [20 mM Hepes, pH 7.9, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.4 mM PMSF, antipain (1 $\mu\text{g}/\text{ml}$), leupeptin (1 $\mu\text{g}/\text{ml}$)],
- 9 Preparing Buffer C: (extraction buffer with salt), [20 mM Hepes, pH 7.9, 400 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.4 mM PMSF, antipain (1 $\mu\text{g}/\text{ml}$), leupeptin (1 $\mu\text{g}/\text{ml}$)],

- 10 Preparing Buffer D: (cytoplasmic extraction clarification buffer), [20 mM Hepes, pH 7.9, 400 mM NaCl, 0.2 mM EDTA, 40% glycerol, 1 mM DTT, 0.4 mM PMSF, antipain (1 μ g/ml), leupeptin (1 μ g/ml)],
- 11 Performing Simultaneous isolation of protein and DNA comprising the steps of,
- 12 Resuspending the cell pellets in 100-125 μ l (2 pellet vol) of Buffer A,
- 13 Maintaining the resuspended cell pellets on ice for 10-15 min with occasional tapping,
- 14 Pelleting the nuclei by centrifuging at 2,000 rpm for 5 min at 4 $^{\circ}$ C,
- 15 Removing the cytoplasmic supernatant fraction to a second Eppendorf tube,
- 16 Quick freezing on dry ice in a -86 $^{\circ}$ C freezer and store for future use,
- 17 Washing the bottom nuclear fraction with 200-300 μ l Buffer B to remove NP-40,
- 18 Centrifuging at 2,000 rpm for 5 min at 4 $^{\circ}$ C,
- 19 Resuspending the pelleted nuclei into 100-130 μ l high salt Buffer C on ice for 45 min and mixing periodically by tapping to extract the nuclear proteins,
- 20 Centrifuging the nuclear fraction in an Eppendorf centrifuge at 13,000 rpm for 15 min at 4 $^{\circ}$ C,
- 21 Removing the supernatants, aliquoted, in 25 μ l and quick freezing on dry ice,
- 22 Storing at -86 $^{\circ}$ C,
- 23 Quick freezing the remaining pellet containing nucleic acids and other debris,
- 24 Storing at -86 $^{\circ}$ C,
- 25 Clarifying the cytoplasmic fraction by adding 1/3 vol of Buffer D to this fraction for 30 min at 4 $^{\circ}$ C to equilibrate the cytoplasmic proteins with NaCl,
- 26 Centrifuging at 13,000 rpm for 15 min.,

- 27 Removing and quick freezing the supernatants and storing at -86°C ,
- 28 Performing DNA extraction and analysis comprising the steps of,
- 29 Thawing frozen cell pellets from on ice for 10 min,
- 30 Adding 100 μl of Buffer [0.1 % SDS, 10 mM Tris-HCl, pH 7.9, 10 mM EDTA, 10 mM NaCl] for 15 min,
- 31 Mixing using wide bore Eppendorf pipet tips,
- 32 Adding RNAase A for 2 h at 37°C with gentle tapping every 30 min,
- 33 Adding proteinase K [200 μl /ml] for 2 h at 37°C with gentle tapping every 30 min,
- 34 Extracting with an equal volume of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) saturated phenol,
- 35 Removing the upper aqueous layer was removed to another tube,
- 36 Performing back extractions twice with 50 μl of TE,
- 37 Collecting the DNA solutions in a fresh tube,
- 38 Adding an equal volume of phenol/chloroform (50:50) mixture,
- 39 Mixing by inverting repeatedly,
- 40 Centrifuging at 3,000 rpm for 10 min,
- 41 Removing the upper aqueous phase,
- 42 Extracting with an equal volume of chloroform/isoamyl (96:4) alcohol,
- 43 Removing the upper aqueous phase containing the DNA and precipitating using 0.5 M NaCl and 3 vol of ice cold ethanol at -20°C ,
- 44 Centrifuging the samples at 13,000 rpm for 30 min,
- 45 Air drying the DNA pellets,

- 46 Dissolving DNA in 300 μ l of 0.1X TE at 37 °C for 4-6 h,
- 47 Digesting ten μ l of the high molecular weight DNA from each sample for 4 h with 30 units of restriction enzymes,
- 48 Electrophoresing on a 0.8% TAE (0.40 M Tris-acetate, 1 mM EDTA) agarose gel,
- 49 Staining the DNA gel with ethidium bromide,
- 50 Photographing with UV light,
- 51 Radiolabeling of the sequence specific oligonucleotides comprising the steps of,
- 52 Synthesizing single stranded oligonucleotides on a DNA synthesizer and annealing with the complimentary strand by combining 4 μ g of both strands in a tube with total volume of 30 μ l of annealing buffer (5 mM NaCl, 10 mM Tris-HCl and 0.2 mM EDTA),
- 53 boiling the tubes for 5 min and slowly cooling to room temperature for 6 h,
- 54 Heating the tubes to 55 °C for 5 min and then cooling on ice for 10 min,
- 55 Quantitating 4 μ l aliquot of the annealed oligos at 260 λ and storing the remainder at -20 °C until radiolabeled,
- 56 Preparing probes by radiolabeling 200 ng of annealed oligonucleotide in 15 μ l of total volume containing 50 μ Ci of [γ - 32 P]ATP (6,000 Ci/mmol), 20 Units of T4 polynucleotide kinase, and 1.5 μ l 10X T4 polynucleotide kinase buffer for 1 h at 37 °C,
- 57 Filling in the 5' over-hang ends with 5 Units of Klenow with 3.0 μ l of 10X Klenow buffer and 0.15 mM each of dATP, dCTP, dGTP, and dTTP for 40 min at 37 °C in a reaction volume of 30 μ l,

- 58 Increasing the volume to 1 ml with sterile TE with 200 mM NaCl, pH 8.0, and the labeled oligonucleotides were purified on a NACS Prepac column, to separate the unincorporated nucleotides,
- 59 Precipitating the labeled, purified oligonucleotides overnight with 3 vol absolute ethanol at -20 °C,
- 60 Centrifuging at 13,000 rpm for 1 h and then vacuum drying,
- 61 Resuspending the labeled oligonucleotide probes in 100 µl of sterile 0.1X TE buffer and storing at -20 °C,
- 62 Electrophoretic mobility shift assay,
- 63 Incubating 5 µg of nuclear or cytoplasmic extract, for each reaction, with 0.2-0.3 ng of [γ - 32 P]ATP labeled oligonucleotide probe containing either NF- κ B sequence (5'-gatccGGGACTTTCCGCTGGGGACTTTCCG-3') or an AP-1 consensus sequence including the PMA responsive element indicated in bold (5'-gatcc**GTGACTCAGCGCG**-3'),
- 64 Adding 3 µg of poly(dI-dC):poly(dI-dC) as a non-specific competitor and incubating with the nuclear extracts for 10 min prior to the addition of the radiolabeled probe,
- 65 Adding antibodies against p65, p50, *c-Fos* or *c-Jun* to the respective binding reactions for supershift assays and incubating at room temperature for 1.5 h, prior to probing with [γ - 32 P]ATP labeled oligonucleotide for an additional 25 min at room temperature,
- 66 Separating the bound complexes on either a 5% ,for supershift assays, or 6% ,for analytical purposes, acrylamide/bis (30:1 ratio) native gel as required and running at 200v for 3.5 h with 0.25X TBE (0.02 M Tris-borate, 0.5 mM EDTA) as running buffer at room temperature,

12 hours,

68 Analyzing the bound and free DNA protein complexes.

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